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Effective chemotherapy of tuberculosis requires rapid assessment of drug sensitivity because of the emergence of multidrug-resistant *Mycobacterium tuberculosis.* Drug susceptibility was assessed by a simple method based on the efficient production of photons by viable mycobacteria infected with specific reporter phages expressing the firefly luciferase gene. Light production was dependent on phage infection, expression of the luciferase gene, and the level of cellular adenosine triphosphate. Signals could be detected within minutes after infection of virulent *M. tuberculosis* with reporter phages. Culture of conventional strains with antituberculosis drugs, including isoniazid or rifampicin, resulted in extinction of light production. In contrast, light signals after luciferase reporter phage infection of drug-resistant strains continued to be produced. Luciferase reporter phages may help to reduce the time required for establishing antibiotic sensitivity of *M. tuberculosis* strains from weeks to days and to accelerate screening for new antituberculosis drugs.

Tuberculosis remains the largest cause of death in the world from a single infectious disease (1) and accounts for as much as 40% of deaths in human immunodeficiency virus (HIV)-coinfected individuals in some developing countries (2). Infection with conventional *M. tuberculosis* can effectively be cured with a combination of antituberculosis drugs. Ominously, multidrug-resistant tuberculosis (MDR-TB) strains have emerged in several countries, with case fatalities ranging from 40 to 60% in immunocompetent individuals and >80% in immunocompromised individuals (3). However, because *M. tuberculosis* has a doubling time of 20 to 24 hours, current methodology does not allow determination of drug susceptibility for 2 to 18 weeks (4, 5), leaving patients, contacts, and health care workers at risk.

Firefly luciferase represents one of the most efficient available biological reporter molecules because it catalyzes the reaction of luciferin with adenosine triphosphate (ATP) to generate photons with a quantum yield of 0.85 photons per molecule of substrate reacted (6). Because of the availability of a variety of sensitive light-detection systems, luciferase has become the standard assay for measuring ATP (7). Since the molecular cloning of its cDNA (8), the firefly luciferase gene has been used directly as a molecular reporter in cells of a variety of animal, plant, and bacterial species (9). We reasoned that the expression of luciferase activity could serve as a sensitive in vivo measure of ATP in mycobacteria and thus allow us to rapidly test cellular viability of *M. tuberculosis* after its exposure to different antimycobacterial agents. The optimal use the gene could be realized if the lucifer-

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ase activity could be measured directly, without the need for lysing of the mycobacterial cells. To ascertain whether the substrate, luciferin, could be transported across the intact mycobacterial cell wall, we cloned the firefly luciferase (*FFlux*) gene downstream of the heat shock protein 60 (*hsp60*) promoter in a mycobacterial extrachromosomal plasmid vector (10) and downstream of the gene 71 promoter of mycobacteriophage L5 (11) in a mycobacterial integrating vector (12) (Fig. 1A). Both luciferase constructs were electroporated into *Mycobacterium smegmatis* (which multiplies ten times faster than *M. tuberculosis*), and luciferase activity was measured in cells grown to logarithmic phase. On addition of

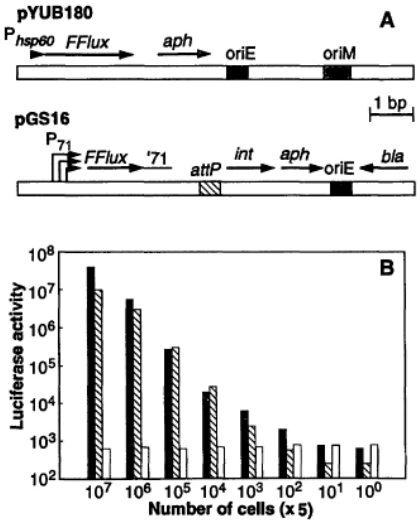

Figure 1. (A) Expression of firefly luciferase in mycobacteria. Schematic of the extrachromosomal plasmid pYUB180 and the integration plasmid pGS16. Abbreviations are as follows: P_{hsp60}, promoter of the BCG *hsp60* gene; P_{71}, promoters of the mycobacteriophage L5's gene 71; *FFlux,* firefly luciferase gene; *aph,* aminoglycoside phosphotransferase that confers kanamycin resistance; oriE, ColE1 origin of replication; oriM, mycobacterial plasmid pAL5000 origin of replication; *attP* and *int,* L5 integration genes; and *bla,* β-lactamase that confers ampicillin resistance to *E. coli.* (B) Sensitivity of *M. smegmatis* cells expressing luciferase. Plasmids pYUB180 and pGSI6 were electroporated into the *M. smegmatis* strain mc^{2}155 (17). Kanamycin-resistant transformants were grown to a density of approximately 5 x 10^8 cells per milliliter, and tenfold serial dilutions were prepared. Samples (100 μl) were mixed with 250 μl of 0.1 M sodium citrate (pH 5) in a 13 by 75 mm polystyrene tube. This mixture was placed in the luminometer (Monolight 2010; Analytical Luminescence Laboratory, San Diego, California), 100 μl of 1 mM luciferin (Sigma, St. Louis, Missouri) was injected into the tube, and the luciferase activity was measured. *Mycobacterium smegmatis* cultures are indicated as follows: mc^{2}155 (pYUB180), black; mc^{2}155 (pGS16), diagonal; and mc^{2}155 cells alone, white.

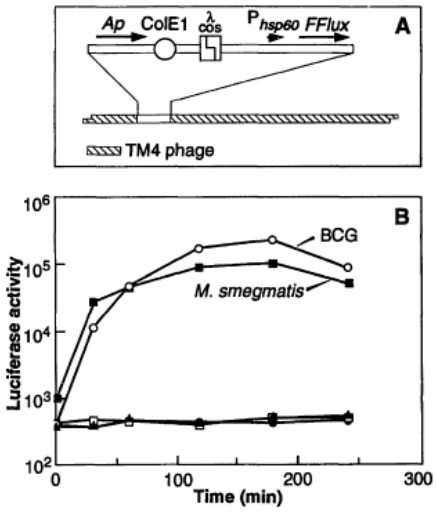

Figure 2. Infection of mycobacteria with LRPs results in the production of light. (A) Schematic of the luciferase reporter mycobacteriophages phAE39 and phAE40. Luciferase reporter phages were made by construction of shuttle phasmids in which an *E. coli* cosmid pYUB216 (18) was inserted into a nonessential region of the mycobacteriophage TM4. The cosmid pYUB216 contains (i) *FFlux* in a transcriptional fusion with the *hsp60* promoter of BCG, (ii) a ColE1 origin and an ampicillin resistance gene *(Ap)* for replication and selection in *E. coli,* (iii) a bacteriophage λ cos sequence, and (iv) a unique Bcl I site. The phAE39 shuttle phasmid was constructed in a manner similar to that described previously (14), with Bcl I-digested pYUB216 ligated to TM4 DNA that had been partially digested by SAU 3AI. The resulting shuttle phasmid, phAE39, like its parent TM4, readily forms plaques of *M. tuberculosis,* but does not efficiently form plaques on BCG. Spontaneous host range mutants of phAE39 could be isolated at frequencies of 10^{-6} to 10^{-7}, and one such mutant was isolated and designated phAE40. (B) Light production in mycobacteria after infection with the luciferase reporter phage phAE40. High titers of phAE40 were prepared as described previously for TM4 phages (19). *Mycobacterium smegmatis,* mc^{2}155 cells, and BCG-Pasteur cells were grown in roller bottles to approximately 5 × 10^7 cells per milliliter in Middlebrook 7H9 broth with ADC enrichment and 0.05% Tween-80 [M-ADC-TW broth (19)] at 37°C. Either the *M. smegmatis* or the BCG cells were harvested by centrifugation and washed twice in M-ADC broth (containing no Tween-80). The resulting pellet was resuspended in the original volume of M-ADC broth. The cells were diluted fivefold into fresh M-ADC broth and were allowed to incubate overnight standing at 37°C. (Tween-80 seems to remove the receptors, and we have found that optimal activities are attained if the cells have a chance to grow in the absence of Tween-80. This procedure possibly allows regeneration of phage receptors.) One milliliter of washed cells (approximately 5 × 10^7 cells) was mixed with 0.1 ml of phAE40 particles [5 × 10^8 plaque-forming units (PFU) per milliliter] that had been concentrated on CsCl gradients to attain a multiplicity of infection of 10. The cell-phage mixture was incubated at 37°C. Beginning at the time of the addition of the phAE40, we removed 0.1-ml samples at the times designated in the graph. Luciferase activity was measured as described in Figure 1 and plotted. Filled circles, BCG cells alone; open circles, BCG cells plus phAE40; filled triangles, mc^{2}155 cells alone; filled squares, mc^{2}155 cells plus phAE40; open squares, phAE40 alone.

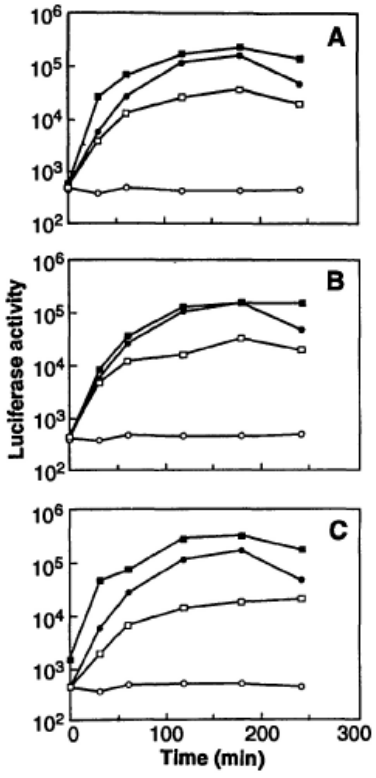

Figure 3. Comparison of the kinetics of light production of drug-sensitive BCG cells to that of drug-resistant BCG mutant cells after phage infection. Spontaneous mutants of BCG-Pasteur strains were isolated on Middlebrook 7H10 agar containing either rifampicin (50 μg/ml), streptomycin (250 μg/ml), or isoniazid (5 μg/ml). The rifampicin-, streptomycin-, or isoniazid-resistant mutants were purified and designated as mc^{2}768, mc^{2}767, and mc^{2}765, respectively. All three mutants and the BCG parent were grown to midlogarithmic phase, harvested, and washed as described in Figure 2 BCG cells (circles) and mutant cells (squares) were incubated in the presence (open symbols) or absence (filled symbols) of rifampicin, streptomycin, or isoniazid. (A) The mc^{2}768 cells and the BCG cells were incubated standing at 37°C in the presence or absence of rifampicin (50 μg/ml) for 24 hours. A 0.5-ml sample (approximately 5 × 10^7 viable cells) was mixed with 0.1 ml (5 × 10^8 PFU) of phAE40 particles, and luciferase activity was determined as described in Figure 2 and plotted as a representative experiment. This experiment was repeated a minimum of three times. The absolute background and peak luminescence signals varied approximately twofold between experiments, but the ratios of signals of drug-resistant relative to susceptible cells were constant (20- to 100-fold depending on the strain). (B) The mc^{2}767 cells and the BCG cells were incubated standing at 37°C in the presence or absence of streptomycin (250 μg/ml) for 24 hours. **(C)** The mc^{2}765 cells and the BCG cells were incubated standing at 37°C in the presence or absence of isoniazid (50 μg/ml) for 24 hours.

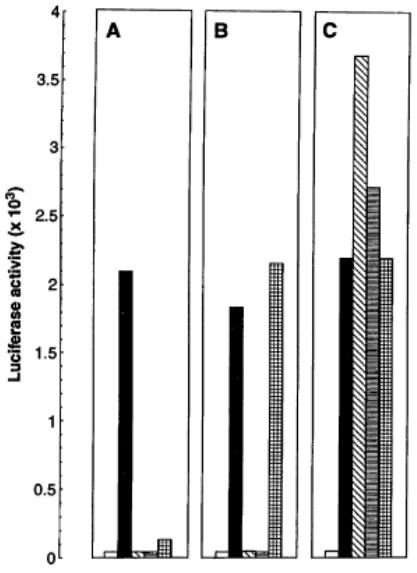

Figure 4. Use of luciferase reporter phage assay to distinguish drug-sensitive from drug-resistant *M. tuberculosis* strains. The following *M. tuberculosis* strains were grown in a biological safety level 3 containment facility: (i) The virulent drug-sensitive *M. tuberculosis* Erdman strain; (ii) strain 92-2025, a singly isoniazid-resistant strain; and (iii) an MDR strain of tuberculosis that has been shown to be resistant to rifampicin, streptomycin, isoniazid, ethambutol, and ethionamide and the cause of several nosocomial outbreaks in New York City (20). The Erdman strain was subcultured from the starter culture by inoculation of 0.4 ml into 20 ml of Middlebrook 7H9 broth containing OADC enrichment (Difco Laboratories, Detroit, Michigan) plus 0.5 Tween-80 (M-OADC-TW broth). The 92-2025 and the MDR strains, which grow more slowly than the Erdman strain, were subcultured by inoculation of 2 ml into 20 ml M-OADC-TW broth. All three cultures were grown standing at 37°C for 7 to 8 days. The cells were washed as described above and 0.5. resuspended in 0.5× the original volume. Washed cells (0.2 ml) were inoculated into 0.7 ml of M-OADC broth (19) and incubated in 13 by 100 mm polypropylene tubes in a heating block in a biohazard hood for 48 hours. Rifampicin, streptomycin, or isoniazid were added to final concentrations of 2 μg/ml, 6 μg/ml, and 1 μg/ml, respectively. After 48 hours of incubation, 0.1 ml of phAE40 particles (1×10^{11} particles) were added to attain a multiplicity of infection of 1000. Samples of 100 μl were removed at 1, 3, and 5 hours after addition of the phage and were mixed with 250 μl of 0.1 M sodium citrate (pH 5) in a Lumacuvette (Lumac BV, Netherlands). One hundred microliters of 1 mM luciferin were added, and the Lumacuvette was plugged with cotton. The tube was placed in a Lumac Biocounter (M1500 P), and readings were recorded as described above. (The Lumac biocounter has dimensions that permit it to fit in a standard biohazard hood.) The light production followed kinetics similar to the BCG experiments, and the readings at 3 and 5 hours differed by no more than twofold. The results at 3 hours are shown for the Erdman (**A**), 92-2025 (**B**), and the MDR (**C**) *M. tuberculosis* strains. A repeated experiment gave similar results, with the samples cultured in the absence of drug exhibiting an 80-fold greater luminescence than the cells cultured with rifampicin or streptomycin and greater than tenfold luminescence relative to those cultured with isoniazid at 3 and 5 hours. Open bars, cells alone; filled bars, cells plus LRPs; diagonal lines, cells plus rifampicin plus LRPs; cross-hatching, cells plus streptomycin plus LRPs; squares, cells plus isoniazid plus LPRs.

luciferin, luciferase activity was readily measured from intact mycobacterial cells infected with both the extrachromosomal and the integrating vectors (Fig. 1B). Serial dilutions indicated that it was possible to detect as few as 500 to 5000 *M. smegmatis* cells expressing firefly luciferase (Fig. 1B), establishing that the luciferase-luciferin system could be developed as a sensitive reporter system for ATP in mycobacteria.

The ability to make use of the luciferase reporter gene to assess drug susceptibilities in clinical isolates requires an efficient means for delivering the luciferase gene into the *M. tuberculosis* cells. Phages offer an ideal vehicle with which to deliver a foreign gene to every cell in a bacterial culture (13). We have previously developed shuttle phasmid vectors from a variety of mycobacteriophages that can be genetically manipulated in *Escherichia coli* and then used to deliver the recombinant DNA into mycobacteria by infection with high efficiency (14). A shuttle phasmid, phAE39, was constructed from mycobacteriophage TM4, which forms plaques on both the fast-growing mycobacterium *M. smegmatis* and the slow-growing mycobacterium *M. tuberculosis,* by insertion of an *E. coli* cosmid into which the *FFlux* gene had been inserted downstream of the strong *hsp60* promoter (Fig. 2A). A host range mutant of phAE39, phAE40, was isolated that was capable of infecting bacillus Calmette-Guerin (BCG) vaccine strains, in addition to *M. smegmatis* and *M. tuberculosis* (Fig. 2A). To test whether these resulting luciferase reporter phages (LRPs) could elicit the production of light after infection of mycobacteria, we mixed the LRPs with *M. smegmatis* cells and then exposed the mixture at different times to luciferin. Light signals could be detected by means of a luminometer within minutes of infection and increased 1000-fold within 2 hours (Fig. 2B). The signals are two to three orders of magnitude lower than an equivalent number of cells harboring the luciferase plasmids, probably as a result of less efficient gene expression or of inhibitory effects of the phage on host cell metabolism or ATP levels. The similarity of the kinetics of light production in *M. smegmatis* and BCG (Fig. 2B) suggests that differences in gene expression may not be the principal determinant in the tenfold slower growth of BCG (or *M. tuberculosis)* relative to *M. smegmatis*.

It then became feasible to test whether the luciferase reporter phages were capable of distinguishing drug-resistant from drug-susceptible organisms. Mutants of BCG were selected that were resistant to rifampicin, streptomycin, or isoniazid (Fig. 3). When wild-type BCG and the mutants were cultured for 24 hours with the antibiotics, the parental strain did not produce any signal, whereas light was produced by the drug-resistant mutants (Fig. 3). Finally, the luciferase reporter phage assay was tested on clinically derived *M. tuberculosis* strains, both singly and multiply drug-resistant (MDR). The results (Fig. 4) established that luciferase reporter phages can rapidly reveal the patterns of drug susceptibility or resistance of *M. tuberculosis* strains. The apparent lower activity of *M. tuberculosis* relative to BCG (Fig. 3) primarily reflects the use of a luminometer with different light unit definition and sensitivity rather than an intrinsic difference between bacterial strains (15).

Because of the emergence of multidrug-resistant strains, it has become increasingly important to rapidly ascertain patterns of drug susceptibility. These observations demonstrate the use of luciferase reporter mycobacteriophages as simple tools for the rapid determination of drug susceptibility profiles of *M. tuberculosis*. It is expected that this methodology will be adapted for use on clinical isolates with a minimum time of culture. This might be achieved by an increase in the sensitivity of the assay or by engineering of better characterized mycobacteriophages, such as L5 [whose complete DNA sequence is known (16)], to permit higher expression of luciferase. The technology could be adapted for use in developing countries either through use of inexpensive luminometers or of sensitive film technology (7). In addition, luciferase phages or *M. tuberculosis* strains expressing luciferase genes may permit rapid screening of drugs for antituberculosis activity.

REFERENCES AND NOTES

1. C J. L. Murray, K. Styblo, A. Rouillon, in *Disease Control Priorities in Developing Countries,* D. T. Jamison and W. H. Mosley, Eds. (Oxford Univ. Press for the World Bank, New York, in press); C. J. L. Murray, K. Styblo, A. Rouillon, *Bull.*

Int. Union Tuberc. **65***, 6* (1990).

2. Y. Abouya *et al., Am. Rev Respir. Dis.* **145,** 617 (1992).
3. *T. R. Frieden et al., N. Engl. J. Med.* **328,** 521 (1993); M. Goble *et al., ibid.,* p. 527; M. D. lseman and L. A. Madsen, *Clin. Chest. Med.* **10,** 341 (1992); M. D. lseman and J. A. Sbarbaro, *Curr. Clin. Top. Infect. Dis.* **12,** 188 (1992).
4. L. Heifets, *Drug Susceptibility in the Chemotherapy of Mycobacterial Infections,* L. Heifets, Ed. (CRC Press, Ann Arbor, MI, 1991), pp. 89–122.
5. *Morbid. Mortal Weekly Rep.* **41**, 507 (1992).
6. J. W. Hastings, T. O. Baldwin, M. Z. Nicoli, *Methods Enzymol.* **57**, 135 (1978).
7. M. A. DeLuca and W. D. McElroy, eds., *Bioluminescence and Chemiluminescence* (Academic Press, New York, 1981), P E Stanley *et al., J. Biolumin. Chemilumin.* **3**, 131 (1989), G. G. G. Thorpe, T. P. Whitehead, R. Penn, L. J. Dricka, *Clin. Chem.* **30**, 806 (1984).
8. J. R. deWet, K. V. Wood, D. R. Helinski, M. DeLuca, *Proc. Natl. Acad. Sci. U.S.A.* 82, 7870 (1985), J. R. deWet, K. V. Wood, M. DeLuca, D. R. Helinski, S, Subramani, *Mol. Cell. Biol.* **7**, 725 (1987).
9. D. W. Ow *et al., Science* **234**, 856 (1986); J. Gould and S. Subramani, *Anal. Biochem.* **175**, 5 (1988); A. J. Palomeres, M. A. DeLuca, D. R. Helinski, *Gene* **81**, 55 (1989); A. G. Dileia *et al., Nucleic Acids Res.* **16**, 4159 (1988), A. R. Brasier, J. E. Tate, J. F. Habener, *BioTechniques* **7**, 1116 (1989); K. V. Wood, Y. A. Lam, W. D. McElroy, *J. Biolumin. Chemilumin.* **5**, 107 (1990); T. Kondo, N. Takahashi, M. Muramatsu, *Nucleic Acids Res.* **20**, 5729 (1992); A. J. Miller, S. R. Short, N. H. Chua, S. A. Kay, *Plant Cell* **4**, 1075 (1992), J. Engebrecht, M. Simon, M. Sherman, *Science* **227**, 1345 (1985); J. J. Shaw and C. J. Kado, *BioTechniques* **4**, 560 (1986), G. Schmetterer, C. P. Wolk, J. Elhai, *J. Bacteriol.* **167**, 411 (1986), O. A Carmi, G. S. A. B. Stewart, S. Ulitzur, J. Kuhn, *J. Bacteriol.* **169**, 2165 (1987).
10. C. K. Stover *et al.*, *Nature* **351**, 456 (1991).
11. M. K. Donnelly-Wu, W. R. Jacobs, Jr., G. F. Hatfull, *Mol. Microbiol.* **7**, 407 (1 993).
12. M. H. Lee, L. Pascopella, W. R. Jacobs, Jr., G. F. Hatfull, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 3111 (1991).
13. S. Ulitzer and J. Kuhn, in *Bioluminescence and Chemiluminescence, New Perspectives,* J. Sclomerick, R. Andresen, A. Kapp, M. Ernst, W. G. Woods, eds. (Wiley, New York, 1987), pp. 463–472; C. P. Kodikara, H. H. Crew, G. S. A. B. Stewart, *FEMS Microbiol. Lett.* **83**, 261 (1991).
14. W. R. Jacobs, Jr., M. Tuckman, B. R. Bloom, *Nature* **327**, 532 (1987); S. B. Snapper *et al.*, *Proc Natl. Acad. Sci. U.S.A.* **85**, 6987 (1988).
15. W. R. Jacobs, Jr., *et al.*, unpublished data.
16. G. F. Hatfull and G. J. Sarkis, *Mol. Microbiol.* **7**, 395 (1993).
17. S. B. Snapper, R. E. Melton, S. Mustafa, T. Kieser, W. R. Jacobs, Jr., *Mol. Microbiol.* **4**, 1911 (1990).
18. R. G. Barletta *et al.*, unpublished data.
19. W. R. Jacobs, Jr., *et al.*, *Methods Enzymol.* **204**, 537 (1991).
20. *Morbid. Mortal. Weekly Rep.,* in press.
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